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Novel Expression Vector for Secretion of Cecropin AD in *Bacillus subtilis* with Enhanced Antimicrobial Activity▽

Xiang Chen,† Faming Zhu,† Yunhe Cao, and Shiyan Qiao*

National Key Laboratory of Animal Nutrition, National Feed Engineering Technology Research Center, China Agricultural University, No. 2 Yuanmingyuan West Road, Beijing 100193, People's Republic of China

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Cecropin AD, a chimeric antimicrobial peptide obtained from cecropins, is effective at killing specific microorganisms. However, a highly efficient expression system is still needed to allow for commercial application of cecropin AD. For the exogenous expression of cecropin AD, we fused the cecropin AD gene with a small ubiquitin-like modifier (SUMO) gene and a signal peptide of SacB, while a *Bacillus subtilis* expression system was constructed based on *Bacillus subtilis* cells genetically modified by the introduction of an operon including an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible Spac promoter, a signal peptide of *amyQ*, and the SUMO protease gene. The recombinant cecropin AD was expressed, and 30.6 mg of pure recombinant protein was purified from 1 liter of culture supernatant. The purified cecropin AD displayed antimicrobial activity against some pathogens, such as *Staphylococcus aureus* and *Escherichia coli*, and was especially effective toward *Staphylococcus aureus*, with MICs of <0.05 μM (0.2 μg/ml). Stability analysis results showed that the activity of cecropin AD was not influenced by temperatures as high as 55°C for 20 min; however, temperatures above 85°C (for 20 min) decreased the antimicrobial activity of cecropin AD. Varying the pH from 4.0 to 9.0 did not appear to affect the activity of cecropin AD, but some loss of potency was observed at pH values lower than pH 4.0. Under the challenge of several proteases (proteinase K, trypsin, and pepsin), cecropin AD maintained functional activity. The results indicated that the recombinant product expressed by the designed *Bacillus subtilis* expression system was a potent antimicrobial agent and could be applied to control infectious diseases of farm animals or even humans.

Antimicrobial peptides are important components of the innate immune defense against microbial pathogens in a wide range of organisms (12, 16). Cecropin A and cecropin D belong to the cecropin family of antimicrobial peptides and were originally isolated from the insect *Hyalophora cecropia* (1, 18). Both compounds exert strong antibiotic activity against gram-positive and gram-negative bacteria at micromolar concentrations (1, 18). Cecropin A also showed potential usefulness against *Pseudomonas aeruginosa* infections (11).

Cecropins consist of a single polypeptide chain of common amino acids and are well suited for economical production through the application of recombinant DNA technology or peptide synthesis (8). The chimeric cecropin AD peptide, which has the first 11 residues from *Hyalophora cecropia* cecropin A and the last 26 residues from *Hyalophora cecropia* cecropin D, was designed to have greater potential to inhibit tested organisms or even tumor cells than previously developed cecropins (10, 46). However, a highly efficient expression system is needed to allow for commercial application of cecropin AD.

Several recombinant cecropins have been expressed in *Escherichia coli* (47) and *Pichia pastoris* (21, 48) expression systems. However, the expression of foreign proteins in *E. coli* often leads to the formation of densely packed denatured peptide molecules in the form of insoluble particles called inclusion bodies. Inclusion body proteins are devoid of biological activity, and an elaborate process involving solubilization, refolding, and purification is needed to partially recover a functionally active product (34). While use of a yeast expression system such as *P. pastoris* may solve the problem of posttranslational modifications (21), it requires a significant investment during the later period of batch cultivation, which renders it uneconomical.

The *Bacillus subtilis* expression system does not have the disadvantages of the two expression systems previously mentioned and can potentially serve as an efficient expression host (44, 45, 49), especially for the secretion of heterologous proteins (36, 40). Moreover, the secreted foreign proteins usually remain in biologically active forms (27), and the downstream purification process is greatly simplified (36, 45). In addition, it has other advantages, such as a theoretically higher yield, no aggregation of the product, and the possibility for continuous cultivation and production (36).

Small ubiquitin-like modifier (SUMO) fusion technologies have been able to improve heterologous expression by overcoming challenges such as proteolytic degradation of target proteins and protein misfolding (7, 17). In this study, a SUMO fusion with cecropin AD was designed to protect the host strain from the antimicrobial's action. Since the special secondary structure of SUMO is recognized and cleaved by SUMO protease, the cleavage would not result in extraneous residues of the target protein, which would therefore yield a native protein (7). In this work, we describe a *B. subtilis* expression system based on *B. subtilis* cells genetically modified

* Corresponding author. Mailing address: National Key Laboratory of Animal Nutrition, National Feed Engineering Technology Research Center, China Agricultural University, No. 2 Yuanmingyuan West Road, Beijing 100193, People's Republic of China. Phone: 86-10-6273-1456. Fax: 86-10-6273-3688. E-mail: qiaoshy@mafic.ac.cn.

† Xiang Chen and Faming Zhu contributed equally to this work.

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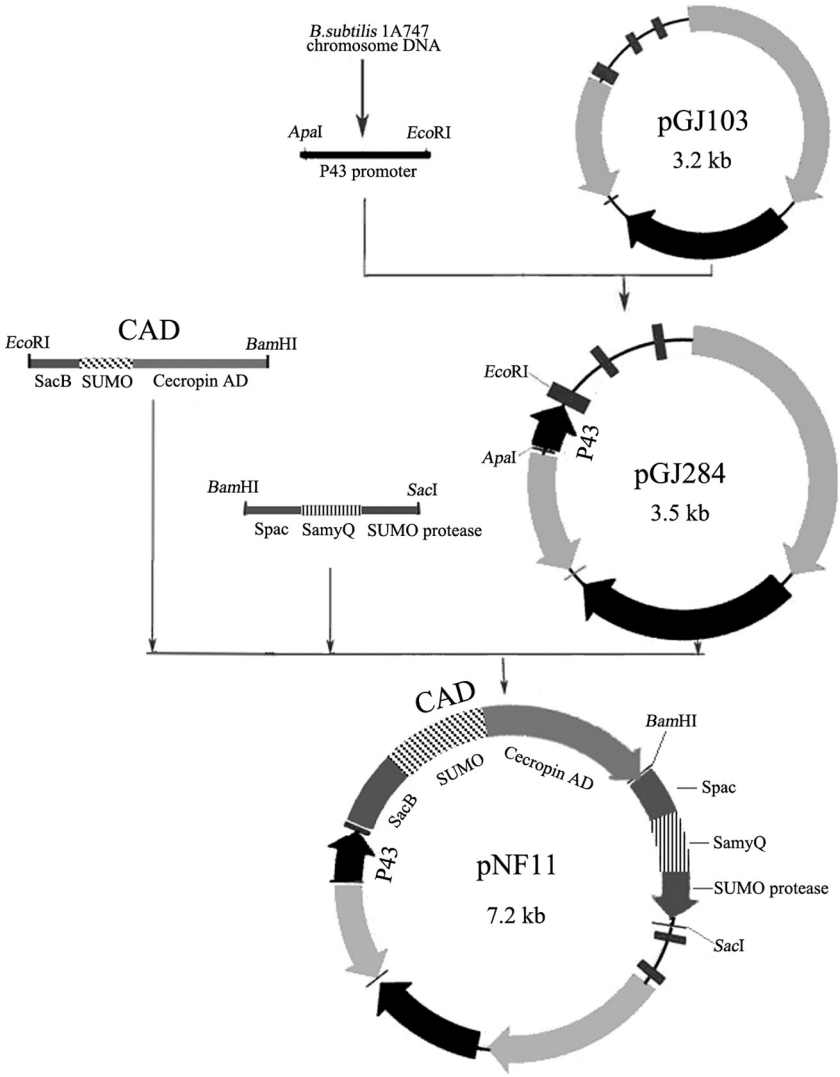

FIG. 1. Construction of recombinant plasmid pNF11. The pNF11 plasmid containing the cecropin AD gene was obtained after three-step construction. SamyQ, signal peptide of *amyQ*.

with a SUMO protease (4) and a two-cistron expression vector gene encoding cecropin AD fused to SUMO.

MATERIALS AND METHODS

Enzymes, chemicals, and antibiotics. T4 DNA ligase, *Taq* DNA polymerase, pUCm-T vector, and all restriction enzymes were purchased from Promega (Madison, WI). The protein marker was obtained from TaKaRa Biotechnology (Shiga, Japan). Antibiotics were purchased from Sigma (St. Louis, MO). All other chemicals used were of the highest grade commercially available.

Bacterial strains and plasmids. The gene encoding the chimeric antibacterial peptide cecropin AD, with the previously reported sequence **KWKLFKKIEKV** GQRVRDAVISAGPAVATVAQATALAK (residues in bold are from cecropin A, and the others are from cecropin D) (10), was synthesized with a SUMO gene and a signal peptide of SacB as a full-length oligonucleotide by standard solid-phase methods at AUGCT Biotechnology Company (Beijing, China). The recombinant plasmid D1778-1, containing the cecropin AD gene and the SUMO gene with a signal peptide of SacB, was supplied by the AUGCT Biotechnology Company (Beijing, China). *Escherichia coli* DH5α and the shuttle vector pGJ103 were obtained from our laboratory (National Feed Engineering Technology Research Center, Beijing, China). *B. subtilis* 1A747 was obtained from the Bacillus Genetic Stock Center (Beijing, China). Preparation of plasmid DNA from *E. coli* cells and transformation of *B. subtilis* were carried out using standard procedures (32).

Plasmid construction and expression. The promoter p43 was amplified from genomic DNA of *B. subtilis* 1A747. After cloning using the pUCm-T vector, an intact plasmid including the p43 promoter was inserted into plasmid pGJ103 (3.2 kb), resulting in pGJ284 (3.5 kb). The plasmid containing the fusion genes of cecropin AD and SUMO with a signal peptide of SacB was digested with EcoRI and BamHI, while pGJ284 was digested by EcoRI and BamHI. The two digested products were linked by T4 DNA ligase, which yielded the recombinant plasmid pNF11 (Fig. 1). The operon, including the inducible Spac promoter, a signal peptide of *amyQ*, and the SUMO protease gene, was inserted between BamHI and SacI sites of plasmid pNF11. Plasmid pNF11 was used for the transformation of *E. coli* DH5α, and the positive transformant was screened at a final concentration of chloramphenicol of 5 μg/ml. The positive clones were further identified by colony PCR with primers F1 (5′-GCGAATTCATGAACA TCAAAAAG-3′) and R1 (5′-GGAGCTCTTAAAGGAGCTGC-3′), followed by a restriction analysis with EcoRI and SacI. The sequence was analyzed by SinoGenoMax Company (Beijing, China).

The plasmid pNF11 was used for transformation of *B. subtilis* 1A747, and positive colonies were selected based on chloramphenicol resistance. The clones were picked out and shaken at 37°C overnight in Luria-Bertani (LB) broth containing 5 μg/ml of chloramphenicol. After this, the culture was inoculated

into new LB broth at a ratio of 1:100, with shaking at 37°C until an optical density of 600 nm of 0.8 to 1.0 was reached. The culture was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sangon, Shanghai, China) and incubated at 37°C for an additional 24 h, with a rotation speed of 250 rpm. The supernatant was then collected by centrifugation. Various pH values of the culture medium (pH 3.0 to 7.0) were tested to find the optimal expression of the recombinant proteins. The optimal medium conditions for the expression of cecropin AD were found at a pH level of 6.5 (data not shown).

Purification of recombinant cecropin AD. The supernatant was firstly filtered twice using an Amicon ultrafiltration device (Millipore, Billerica, MA). The supernatant, which contained proteins ranging from 3 to 10 kDa, was dialyzed overnight in 0.1 M sodium acetate and then applied to a CM-Sepharose CL-6B column (Pharmacia Biosciences, Piscataway, NJ) preequilibrated with 0.1 M sodium acetate (pH 5.0). The column was washed with 0.1 M acetate buffer, and the proteins were eluted with a linear gradient of 0.1 to 1.0 M sodium acetate (pH 5.0). A sample (100 μl) of the filtrate was collected, subjected to semipreparative reversed-phase high-performance liquid chromatography (HPLC) on a C_{18} column (250 mm by 4.6 mm by 5 μm by 300 Å), and equilibrated in 0.1% trifluoroacetic acid and 18% acetonitrile. The bound protein was eluted with a linear gradient of acetonitrile (18 to 45% [vol/vol]) in 0.1% trifluoroacetic acid. The flow rate was 1.0 ml/min, and the absorbance of the eluate was monitored at 280 nm. The peak of chimeric cecropin AD eluted at 31% acetonitrile from the reversed-phase HPLC column was determined by analysis of fractions by Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) (33). The fractions containing cecropin AD were collected for the next step. The purity of the recombinant cecropin AD was determined by electrospray ionization mass spectrometry (Agilent Technologies, Foster City, CA).

Antimicrobial activity. (i) MIC determination. The indicator bacteria *Escherichia coli* K12D31, *E. coli* IVDC C83901 (serotype O8:K87, K88ac), *E. coli* IVDC C83529 (serotype O141:K99), *Salmonella enterica* serovar Enteritidis IVDC C79-52, *Salmonella enterica* serovar Typhimurium IVDC C77-31, *Staphylococcus aureus* IVDC C56005, and *Streptococcus faecalis* IVDC C55614 were purchased from the China Veterinary Culture Collection Center (Beijing, China). The bacteria were cultivated and incubated in LB medium (0.5% NaCl, 0.5% yeast extract, 1% tryptone, pH 7).

The MIC was determined in sterilized 96-well plates in a final volume of 200 μl, using a microdilution assay as described previously (31). A stock solution of the peptides was diluted 10-fold with culture medium and 100 μl of LB medium. The bacteria (2×10^7 to 4×10^7 cells/ml) were added to 100 μl of the LB medium-peptide solution (in serial twofold dilutions). Growth was determined by taking optical density measurements at 620 nm. The MIC was the lowest peptide concentration that showed no increase in optical density after overnight incubation at 37°C.

(ii) Assessment of stability. Assays of the antibacterial activity of cecropin AD at different temperatures (37, 45, 55, 65, 75, 85, and 95°C for 20 min) and different pH values (pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0) were performed to assess the stability of cecropin AD under different biochemical conditions. The assays were conducted as described previously (25, 39), with slight modifications. LB agar inoculated with indicator bacteria (approximately 10^6 viable cells per 10 ml) was poured over plates that had been fitted with Oxford cups to make wells. Five-micromolar peptide samples that were previously treated under various conditions were added to the wells. The agar overlays were incubated overnight at 30°C, and the inhibition zones were recorded.

The susceptibility of cecropin AD to proteinase K, trypsin, and pepsin (10 μg/ml in phosphate-buffered saline) was assessed by adding it to a solution of the peptides in phosphate-buffered saline (1 μM) (28). The reaction was monitored as described above. *S. aureus* IVDC C56005 was used as the indicator strain.

Circular dichroism spectroscopy analysis. Circular dichroism spectroscopy analysis of chimeric cecropin AD was carried out in a Jasco 810 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan). The purified cecropin AD sample was used at 6 mg/ml in 100 mM phosphate buffer (pH 8.0) and in the presence of small unilamellar vesicles for UV circular dichroism measurements, using a 1-mm-path-length cuvette at room temperature. Diphosphoryl lipid A (Sigma, St. Louis, MO) was prepared as described before (9), with the following small modification: diphosphoryl lipid A stock solution (100 μM) was prepared by dissolving the appropriate amount of diphosphoryl lipid A in 10 mM sodium phosphate buffer (pH 8.0). The preparation of palmitoyl-oleoyl-phosphatidylglycerol liposomes (Sigma, St. Louis, MO) was performed by a previously described method (41). The circular dichroism spectrum was presented as an average of scans recorded from 250 nm to 190 nm at a rate of 50 nm/min. Information on the cecropin AD secondary structure was deduced by a previously described method (15, 22).

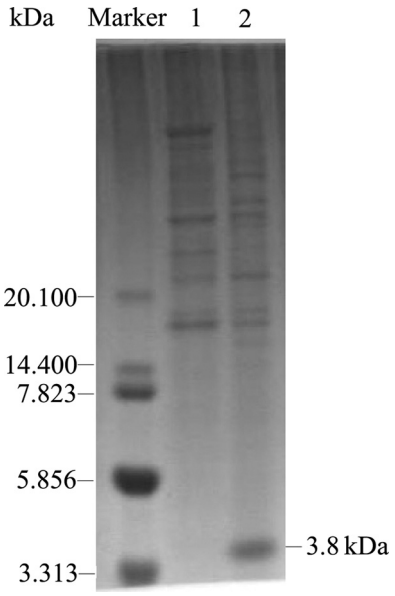

FIG. 2. Expression of cecropin AD in *Bacillus subtilis.* SDS-PAGE analysis was performed with cecropin AD expressed in *Bacillus subtilis* 1A747. Lane 1, total cell protein, uninduced; lane 2, total cell protein, induced; marker lane, low-range molecular mass marker (TaKaRa, Shiga, Japan). The position of recombinant cecropin AD is indicated.

Secondary structural predictions. Neural network-based secondary structural predictions for the helical peptides were carried out online (http://www.predictprotein.org) with the PHD Predict Protein program (29, 30). The hydrophobicity was analyzed using an online Kyte-Doolittle model program (http://www.expasy.org/cgi-bin/protscale.pl). A nine-residue amino acid sequence window, corresponding to the length of an average helical segment in the protein, was used to identify amphipathic helical motifs.

RESULTS

Plasmid construction and expression. As shown in Fig. 1, the constitutive expression vector pNF11, containing an operon including the IPTG-inducible Spac promoter, a signal peptide of *amyQ*, and the SUMO protease gene, was completed. *B. subtilis* supplied a favorable base for expression, and after three-step construction, a recombinant plasmid containing the fusion gene of cecropin AD and SUMO was transformed into *B. subtilis* 1A747 for expression.

During the initial stage of expression, the chimeric cecropin AD-SUMO protein had no antimicrobial activity in order to prevent toxicity in *B. subtilis* and to allow continuous expression and elevated expression after induction with IPTG. Adjustment of the culture conditions released the SUMO protease and resulted in activation of cecropin AD. As shown in Fig. 2, cecropin AD was expressed at high levels and released directly into the culture medium.

Purification of modified cecropin AD. Following highly efficient purification including ultrafiltration and reversed-phase HPLC, 30.6 mg of pure chimeric antibacterial cecropin AD was obtained from 1 liter of culture medium. Analysis of the purified cecropin AD by Tricine-SDS-PAGE revealed that the molecular mass of cecropin AD was about 3.8 kDa (Fig. 2). Electrospray ionization mass spectrometry analysis of purified

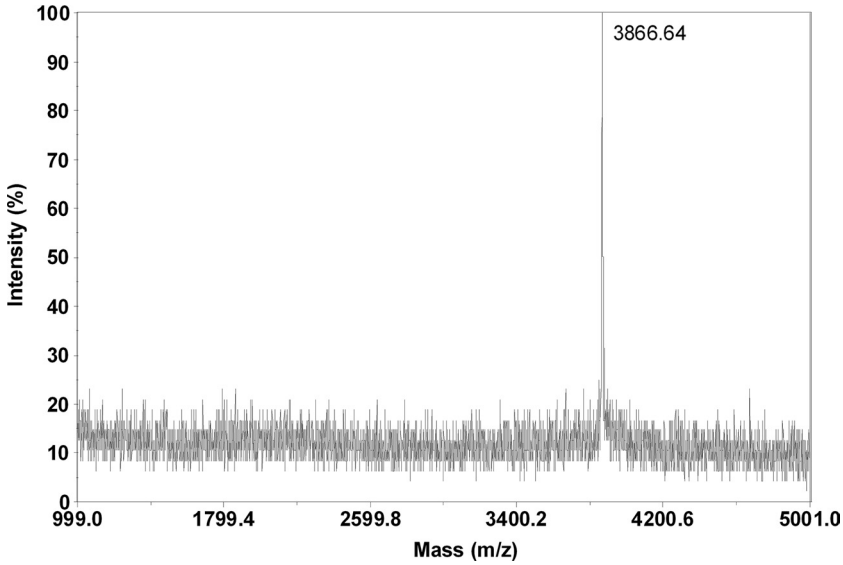

FIG. 3. Analysis of purified recombinant cecropin AD by electrospray ionization mass spectrometry.

cecropin AD demonstrated a single, nondispersed signal with a molecular mass of 3.866 kDa (Fig. 3).

Antimicrobial activity. Serial dilutions of purified cecropin AD were incubated with bacteria, and MICs were determined (Table 1). Cecropin AD was highly effective and had low MICs against all of the gram-negative bacteria (*E. coli* K12D31, *E. coli* IVDC C83901, *E. coli* IVDC C83529, *S. enterica* serovar Enteritidis IVDC C79-52, and *S. enterica* serovar Typhimurium IVDC C77-31) and gram-positive bacteria (*S. aureus* IVDC C56005 and *S. faecalis* IVDC C55614) tested (Table 1). Cecropin AD showed its highest activity against *S. aureus* (MIC, 0.05 μM).

The bactericidal potency of cecropin AD was tested under various incubation conditions, as summarized in Fig. 4A, B, and C. The results of the thermal stability test showed that temperatures as high as 55°C for 20 min had no influence on the activity of cecropin AD; however, temperatures above 85°C (20 min) decreased the antimicrobial activity of cecropin AD by over 50%. Varying the pH from 4.0 to 9.0 had no effect on the activity of cecropin AD, but some loss of potency was observed at pH values lower than pH 4.0. Under the challenge of several proteases (proteinase K, trypsin, and pepsin), cecropin AD maintained a portion of functional activity, as shown in Fig. 4C. When various concentrations (0.01 to 200 μg/ml) of cecropin AD were incubated with human red blood cells for 1 h, no hemolytic activity was observed, indicating that cecropin AD was not toxic to human red blood cells (data not shown).

TABLE 1. MICs of cecropin AD for different bacteria

Strain	MIC (μg/ml)[a]
E. coli K12D31	1.8
E. coli K88	2
E. coli K99	2
Salmonella enterica serovar Typhimurium	8
Salmonella enterica serovar Enteritidis	16
Staphylococcus aureus	0.2
Streptococcus faecalis	24

[a] All MIC determinations were done twice.

Circular dichroism spectroscopy analysis. The secondary structure of chimeric cecropin AD in water solution was determined by UV circular dichroism spectroscopy (Fig. 5). Circular dichroism measurements confirmed that cecropin AD adopted a basically linear, α-helix conformation. Neural network predictions suggested that recombinant cecropin AD contains predominantly α-helices (78%), with some random coils (21%) but no β-sheets. The helical wheel shown in Fig. 6 demonstrates similar results to the circular dichroism measurements.

DISCUSSION

The introduction of SUMO into the fusion gene creatively afforded the advantages of SUMO fusion technologies, but also the SUMO protease was efficient and accurate and did not result in extraneous residues at the N terminus of the chimeric cecropin AD peptide. With the aid of the special operon, the production of cecropin AD in *B. subtilis* was achieved without any later process or damage to the host strain.

The production of antimicrobial peptides such as cecropins with a high efficiency is a significant challenge in developing commercial products. The cecropin protein Mdcec was successfully expressed in the methylotrophic yeast *P. pastoris* (21) and in *E. coli* (47), with 1.2 mg pure recombinant Mdcec obtained from 100 ml of crude yeast extract (21) and 11.2 mg pure active recombinant Mdmcec obtained from 1 liter of *E. coli* culture (47). In this study, the *B. subtilis* expression system for the constitutive expression of recombinant cecropin was adopted. In order to facilitate the expression of cecropin AD, this system included the SUMO protease gene, which could be released under appropriate conditions, thus ensuring the cleavage of the target gene (4). In addition, the modified expression system required no inducer during the process of expression (21), and it produced no inclusion bodies (34, 47). Therefore, the system had the advantage of a high efficiency of expression

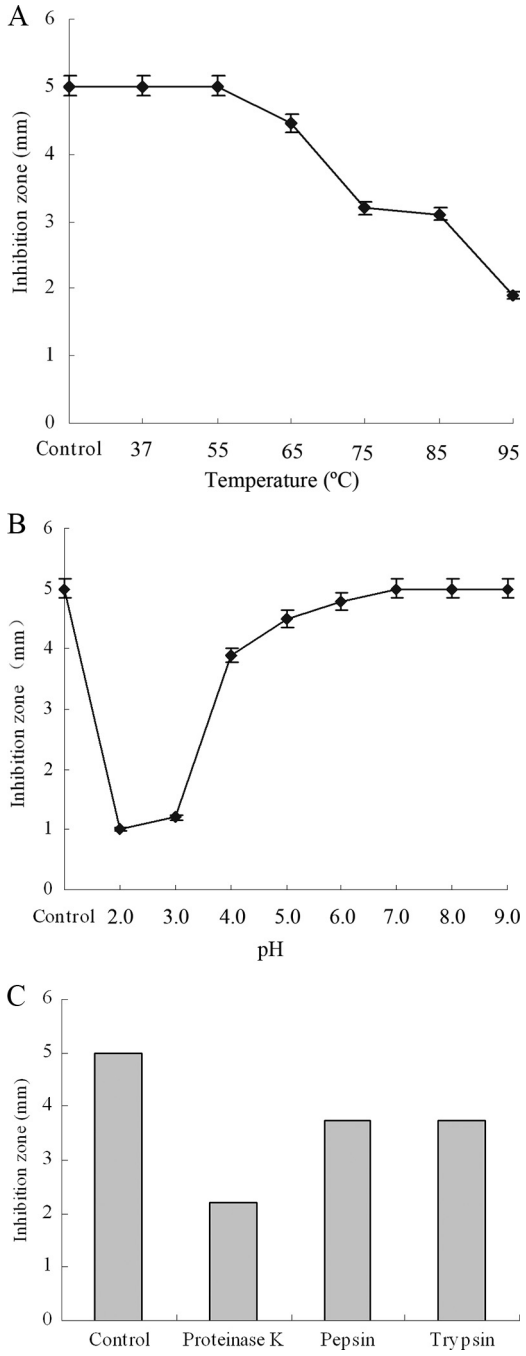

FIG. 4. Effects of temperature (A), pH (B), and proteases (C) on cecropin AD. (A) The peptide sample kept at 4°C was used as a control. (B) The sample kept in the original culture (pH 6.5) was used as a control. (C) The original sample without any enzymatic treatment was used as a control. The graphs were derived from average values for three replicate experiments and almost identical triplicate sets of data. Error bars show standard deviations.

at a low cost and was easy to operate, making it applicable to industrial production. In the present study, the recombinant cecropin AD peptide was expressed at a high level of up to 30.6 mg/liter in bacterial cell cultures, which was higher than the levels of Mdmcec in *E. coli* and Mdcec in *P. pastoris*.

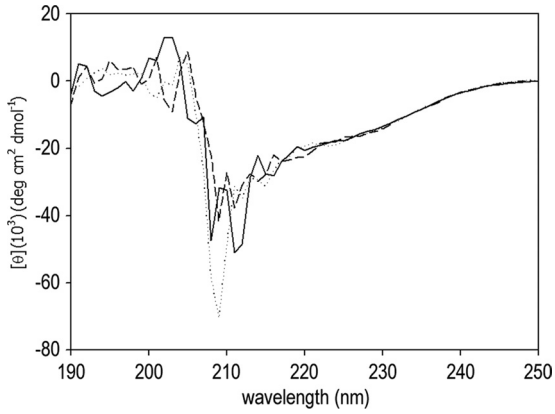

FIG. 5. Circular dichroism spectrometry. The spectra were measured in phosphate buffer (dotted line), in the presence of diphosphoryl lipid A (solid line), and with liposomes (dashed line) and are described in the text.

Tricine-SDS-PAGE analysis of purified cecropin AD revealed that the molecular mass of cecropin AD was about 3.8 kDa, consistent with the theoretical molecular mass of 3.866 kDa obtained by electrospray ionization mass spectrometry. Our measurements confirmed that cecropin AD adopted a basically linear, α-helix conformation, which was consistent with previous findings (13). Neural network predictions suggested that recombinant cecropin AD contained predominantly α-helices (78%), with some random coils (21%) but no β-sheets. Based on all the data, the α-helices seemed to aggregate in two terminals of the sequence and formed a positively charged NH_2-terminal helix, a hydrophobic COOH-terminal helix, and an intervening hinge part, which were the structural basis for its activity. These secondary structures were similar to those in native cecropins (5, 6, 13, 20, 35). Evidence has demonstrated that cecropins bind to negatively charged membrane lipids and form ion channels. The observation of two distinct conduction states (0.4 and 1.93 nS) for cecropin AD (10) also

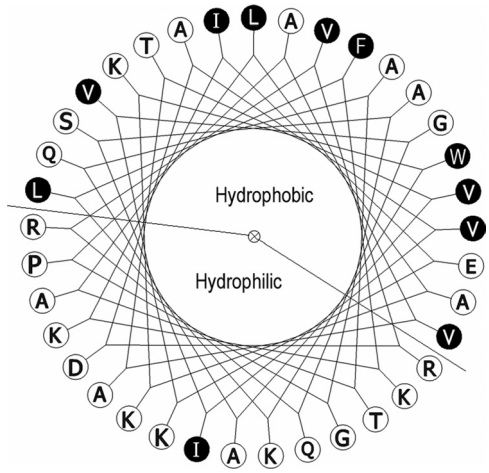

FIG. 6. Helical wheel plot for chimeric cecropin AD. The hydrophobic residues are white with a black background, and the hydrophilic residues are black with a white background. Lines divide the helix into hydrophobic (shaded residues) and hydrophilic parts.

suggested that an ion channel structure was formed for this chimeric peptide.

The results showed that cecropin AD had low MICs against various bacteria tested, including three strains of *E. coli*, two strains of *Salmonella*, *S. aureus*, and *S. faecalis*. Against *S. aureus*, cecropin AD had nearly 40-fold more antimicrobial activity than those of both Mdcec expressed from *P. pastoris* (21) and Mdmcec expressed from *E. coli* (47). With *E. coli*, cecropin AD showed similar inhibitory activity with CA-Hse-NH-Et-NH_2, which was modified in the C terminus of cecropin A (8). Several theories have been proposed to explain the antimicrobial action of cecropins, including the possibility that cecropins form partially selective ion channels (10) or that cecropins bind to negatively charged membrane lipids and form a closely packed layer or "carpet" which renders membranes permeable (14, 38). The structural analysis of purified cecropin AD (Fig. 5 and 6) confirmed the formation of an amphipathic α-helix by N-terminal amino acids 1 through 11 of cecropin A, which is thought to be critical for the activity of cecropins (8). From the results of the antibacterial assays, for some bacteria recombinant cecropin AD showed more effective bactericidal activity than those of cecropins A, B, and D (18) and some other cecropins that have been reported (21, 34, 48).

Currently, antibiotics are widely used as therapeutic agents and growth stimulants for farm animals. However, due to concerns about residues in animal products and the development of bacterial resistance to antibiotics (2), the potential exists for the implementation of a complete ban of the use of antibiotics in animal feed. As a consequence, the development of alternatives to antibiotics has received considerable attention (42). The fact that cecropin AD had significant antimicrobial action against the specific organisms tested suggests that one potential application for the use of cecropin AD may be in the livestock industry, since bacteria such as staphylococci, *E. coli*, and salmonella are some of the primary organisms that cause infectious diseases in livestock (3, 19, 26). Moreover, the fact that cecropin AD has shown potent antitumor activity (46) indicates that it has more extensive and profound applications in medical therapy.

If an application for humans or livestock is to be found for cecropin AD, it is important to test for interference factors against its activity. The finding of high activity in the temperature range of 37 to 55°C is promising for application in humans or farm animals, where the body temperature in which the product must function is close to 40°C for both pigs (43) and poultry (23). However, the reduction in activity when the temperature exceeded 65°C may be unacceptable for withstanding the high temperatures to which feed is exposed during pelleting. Fortunately, most pig rations are not pelleted, but future studies should focus on how to increase the thermostability of the product by introducing disulfide bonds or lanthionine bridges.

It is also important that the compound function in the pH range found in the gastrointestinal tracts of animals. The results indicated that cecropin AD has significant activity in the pH range from 4.0 to 9.0. Snoeck et al. (37) measured the pH in the pig gastrointestinal tract 1 week after weaning and reported pH values ranging from 1.6 to 4.4 in the stomach, 3.2 to 5.8 in the small intestine, and 5.9 to 6.5 in the large intestine. Therefore, cecropin AD would appear to be ideally suited for a role in controlling infectious organisms within the pH range found in the gastrointestinal tracts of recently weaned animals. However, it would inevitably lose some activity during passage through the stomach, which could be ameliorated by some processing technologies, such as coating.

Another factor that could interfere with activity during passage through the gastrointestinal tract would be gastric enzymes and pancreatic enzymes, such as pepsin and trypsin, respectively (24). Our results indicated that cecropin AD maintained activity when it was exposed to these enzymes. A significant reduction in activity was observed when cecropin AD was exposed to proteinase K. Although similar research about the stability of cecropins has not previously been reported, the reduction of antimicrobial activity was obviously seen in *Streptococcus bovis* HC5 when it was exposed to proteinases, peptidases, and heat (25), which shows the common problem of the susceptibility of antimicrobial peptide activity. However, proteinase K is not secreted in the gastrointestinal tracts of animals but is commonly used in molecular biology to digest proteins and to remove contamination from preparations of nucleic acids.

In summary, a *B. subtilis* expression system for cecropin AD was firstly developed based on *B. subtilis* cells genetically modified with a SUMO protease and a two-cistron expression vector gene encoding cecropin AD fused to SUMO. The purified cecropin AD displayed antibacterial activity against both gram-positive and gram-negative bacteria. Antibacterial activity was present at the temperature and pH ranges commonly found in the animal body. In addition, cecropin AD resisted partial degradation by protease enzymes. These findings indicate that the *B. subtilis* expression system could be applied as a powerful tool for large-scale production of cecropin AD, which is expected to become a useful antimicrobial or even therapeutic agent for animals or possibly humans.

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